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TOPIC HIGHLIGHT

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Circadian clock circuitry in colorectal cancer

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Abstract

Colorectal cancer is the most prevalent among digestive system cancers. Carcinogenesis relies on disrupted control of cellular processes, such as metabolism, proliferation, DNA damage recognition and repair, and apoptosis. Cell, tissue, organ and body physiology is characterized by periodic fluctuations driven by biological clocks operating through the clock gene machinery. Dysfunction of molecular clockworks and cellular oscillators is involved in tumorigenesis, and altered expression of clock genes has been found in cancer patients. Epidemiological studies have shown that circadian disruption, that is, alteration of bodily temporal organization, is a cancer risk factor, and an increased incidence of colorectal neoplastic disease is reported in shift workers. In this review we describe the involvement of the circadian clock circuitry in colorectal carcinogenesis and the therapeutic strategies addressing temporal deregulation in colorectal cancer.

Key words: Colorectal cancer; Circadian rhythm; Clock gene

Core tip: The biological clock drives crucial cell processes, such as growth, proliferation, differentiation and apoptosis, controls metabolic pathways, and regulates tissue functions and behavioral cycles. Derangement of these phenomena is involved in colorectal carcinogenesis. The circadian clock circuitry is a leading actor in physiological regulation, a drawn in bystander in colorectal tumorigenesis, and a possible therapeutic target.

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INTRODUCTION

Digestive system cancers account for approximation

20% of neoplastic disease incidence, and colorectal cancer (CRC) is the most prevalent, representing > 50% of all these cancers. CRC is the third most commonly diagnosed cancer, representing approximation 10% of all newly diagnosed cancers apart from skin cancers, with 19.4 new cases per 100000 of the male population and 17 new cases per 100000 of the female population. CRC is the fourth leading cause of cancer deaths, accounting for 8.1% of all cancer-related deaths worldwide. Epidemiological data report a 5-year CRC prevalence of 66.3 cancer survivors per 100000 population worldwide, and a cumulative CRC risk in persons aged < 75 years of 1.96% worldwide; 2.35% in men and 1.62% in women[1,2]. At present, the first-choice treatment is surgical excision with or without adjuvant chemotherapy, and the identification of novel biomarkers for prognosis and molecular targets for therapeutic intervention is urgently needed. CRC onset and progression are related to mutational pathways, such as chromosomal instability and microsatellite instability (MSI). MSI is distinguished as H (high frequency or probability), L (low frequency or probability) and S (stable); is determined by defects in the normal DNA damage response and mismatch repair process, leading to the loss or gain of repeated units on the daughter strand and resulting in length variation[3,4]; and influences cancer-specific survival and time to recurrence in CRC patients[5,6]. An early step in colorectal carcinogenesis is represented by mutations in the gene adenomatous polyposis coli (*APC*), causing large deletions in the C terminus of the proteins that are found in > 80% of human colon cancers. This region is involved in the binding of β-catenin and axin, and its deletion leads to post-translational stabilization and accumulation of β-catenin. In turn, this transcriptional factor translocates from the cytoplasm to the nucleus, where it relieves T-cell factor (TCF)/lymphocyte enhancer factor (LEF)1-mediated transcriptional repression, to activate several oncogenes and other gene targets, such as *c-MYC* and *CCND1*, which stimulate target-gene expression and eventually tumor phenotype development[7,8].

Circadian clock circuitry and the bowel

Colorectal carcinogenesis is related to the progressive loss of homeostatic control of cell proliferation, differentiation and apoptosis. Cellular processes and functions in living organisms show time-related variations[9-11]. The patterns of oscillation may be rhythmic with a period of approximation 24 h and are called circadian[12]. The circadian timing system responsible for the generation of these rhythmic variations is composed of central and peripheral oscillators[13]. The central pacemaker is located in the suprachiasmatic nuclei of the brain, which are entrained to the environmental light-dark cycle by photic inputs conveyed by the retino-hypothalamic tract, and synchronize self-sustained oscillators in peripheral tissues through autonomic nervous system fibers and hormone output; mainly represented by cortisol and melatonin[14,15]. The molecular clock responsible for the generation of circadian rhythmicity consists of a set of interlocking transcription-translation feedback loops that complete one cycle each day and are driven by the core clock genes encoding the circadian proteins brain and muscle aryl-hydrocarbon receptor nuclear translocator-like (ARNT)l (BMAL1/2 also called ARNTL/2), circadian locomotor output cycles kaput (CLOCK) or its paralog neuronal PAS domain protein (NPAS)2, PERIOD (PER) 1-2, CRYPTOCHROME (CRY) 1-2[16]. Other proteins that in some way are related to the clock gene machinery are represented by PER3, TIMELESS, timeless-interacting protein (TIPIN), and protein kinases. The genes *PER1-2* and *CRY1-2* are transcriptionally activated by the basic helix-loop-helix/PAS (period, aryl-hydrocarbon-receptor, single minded) transcription factors CLOCK and BMAL1, which heterodimerize and bind to E-box enhancer elements in the promoters of these genes[16]. In turn, PER and CRY proteins form a repression complex that translocates back into the nucleus and interacts with CLOCK and BMAL1, hindering their activity. *PER3* is believed to be the product of one of the output genes, more than a core clock gene, considering that Per3 knockout mice do not show any circadian phenotype, whereas *PER1* and *PER2* play an essential role in the molecular clockwork[17]. TIMELESS is the homolog of a core circadian gene of *Drosophila melanogaster* and is maintained in mammals, but its role in the function of the mammalian molecular clock is still unclear. TIMELESS and its partner TIPIN interact with components of the DNA replication system to regulate DNA replication processes under both normal and stress conditions and are essential for ataxia telangiectasia and Rad3-related (ATR)-checkpoint kinase (Chk)1 and ataxia telangiectasia mutated (ATM)-Chk 2-mediated signaling and S-phase arrest[18,19]. CLOCK-BMAL1 heterodimer activates an assisting loop that promotes expression of the nuclear receptors reverse transcript of erythroblastosis gene (REV-ERB) α/β (encoded by *NR1D1* and *NR1D2*, respectively), and retinoic acid-related (RAR) orphan receptor (ROR)α/γ, which in turn compete for ROR-responsive elements (ROREs) of the *BMAL1* promoter, and control negatively and positively the rhythmic transcription of *BMAL1*, as the REV-ERBs repress *BMAL1* transcription, while RORs activate it. This stabilizing negative loop is important for precise control of the circadian pacemaker, and these nuclear receptors regulate a number of physiological functions, including circadian rhythmicity, lipid metabolism, and cellular differentiation[20-23]. The correct functioning of the clock gene machinery relies on post-translational modifications of circadian proteins, represented by phosphorylation, O-GlcNAcylation, SUMOylation, acetylation, and deacetylation. Phosphorylation is operated by protein kinases, such as casein kinase (CK)1-ε (encoded by *CSNK1E*), which targets the proteins BMAL1, PER1, PER2, and CRY1; CK2, which targets BMAL1 and PER2; AMP-activated kinase (AMPK), which targets the CRY proteins; and glycogen synthase kinase (GSK)-

3β, which targets the proteins CLOCK, BMAL1, PER2 and CRY2[24]. In the absence of GSK-3β-mediated phosphorylation BMAL1 becomes stabilized, decreasing the dependent circadian gene expression[25]. In turn, GSK-3β regulates the activity of O-GlcNAc transferase, which works as a metabolic sensor gauging the nutrient flux into the hexosamine biosynthesis pathway, and fine-tunes the regulation of the circadian clock through reversible change of structure and transcriptional activity of the circadian proteins CLOCK and PER, as well as stabilization of BMAL1 and CLOCK as a result of inhibition of their ubiquitination[26,27]. Furthermore, BMAL1 is SUMOylated *in vivo* on Lys259; CLOCK is necessary to stimulate this post-translational modification; and BMAL1 SUMOylation and activation oscillate with circadian rhythmicity in the mouse liver[28,29]. Acetylation is operated by CLOCK, whereas deacetylation is operated by SIRT1, a type Ⅲ NAD^+-dependent histone/protein deacetylase that is required for high-magnitude circadian transcription of several proteins encoded by core clock genes, including *BMAL1*, *PER2*, and *CRY1*, which counterbalances the histone acetyltransferase activity of CLOCK, and promotes the deacetylation and degradation of PER2. The interaction of SIRT1 with CLOCK:BMAL1 is not time dependent, whereas the NAD^+-dependent SIRT1 activity changes in a circadian manner, and the circadian regulation of SIRT1 activity depends on CLOCK:BMAL1-mediated regulation of expression of nicotinamide phosphoribosyltransferase (NAMPT); the rate-limiting enzyme involved in NAD^+ synthesis[30-34]. The clock gene machinery controls the expression of hundreds of genes (clock-controlled genes) that drive the expression of proline-and acidic amino acid-rich domain basic leucine zipper (PAR bZIP), including DBP (albumin D-site binding protein), TEF (thyrotroph embryonic factor), HLF (hepatic leukemia factor), and the nuclear factor, interleukin-3-regulated protein (NFIL3, also known as E4BP4), whose promoter contains a RORE and is transcriptionally regulated by REV-ERBs[35]. Many physiological processes in the gastrointestinal tract, such as motility of gut sections, activity of mucosal enzymes, function of mucosal transporters, and proliferation rate of different cell types, exhibit diurnal rhythms[36,37]. Studies in animal models and observations in humans have demonstrated that a circadian biological clock operates in the gastrointestinal tract, and the core clock genes drive the circadian expression of clock-controlled genes and tissue-specific output genes[38,39], coding for proteins involved in gut functions, such as *NHE3*, encoding an electroneutral Na^+/H^+ exchanger, and *ATP1A1*, *ENaCg*, *DRA*, *AE1* and *NHERF1*, involved in colonic NaCl absorption[40]. In addition, the clock gene machinery drives the circadian rhythmicity of gut physiology and motility[41,42], suggesting possible links between disruptions of circadian biology and diseases of the gastrointestinal tract, such as motility disorders, inflammation, and cancer[43,44].

The biological clock in colorectal carcinogenesis

The clock genes drive the circadian rhythmicity of expression of so-called clock-controlled genes, which represent 5%-20% of the genome, comprising key cell-cycle regulators, tumor suppressor genes and oncogenes, and their expression regulates timing of basic cell functions, such as metabolism, xenobiotic detoxification, DNA damage repair, and autophagy[45,46]. Cell-cycle progression is tightly regulated and the circadian system is involved in the control of cell proliferation and apoptosis, driving the transcription and/or post-translational modification of key proteins that are essential for DNA replication such as thymidylate synthase, and molecules that gate cell division in mitosis such as WEE-1[47-49]. Disruption of the circadian clock may lead to deregulated cell proliferation and deregulation of the circadian clock has been implicated in CRC[49-52]. The alteration of bodily temporal organization, defined as circadian disruption, is considered a cancer risk factor, and an increased incidence of CRC has been reported in shift workers by epidemiological studies[53-55].

The role of the disruption of the molecular clockwork in colorectal carcinogenesis and CRC progression is hinted by experimental data showing distortion of circadian regulation in colonic neoplastic tissue and gene-specific disruption in the matched nontumorous tissues. Overexpression of *PER1* in human cancer cell lines results in reduced colony formation and clonogenic expansion, in sensitization to radiation-induced apoptosis, and in altered expression of transcriptional target genes such as c-*MYC* and *p21*. In contrast, *Per2*-null mice show an increase in hyperplasia and neoplasia in response to γ radiation, and *Per2* mutation has been shown to accelerate intestinal polyp formation in $Apc^{Min/+}$ mice[56-58]. PER1 and PER2 are involved in ATM-Chk1/Chk2 DNA damage response pathways and modulate β-catenin, encoded by the clock-controlled gene *CTNNB1*, whose promoter shows BMAL1 occupancy, indicating direct circadian regulation by this transcription factor[59,60], and that can function as an oncogene, influencing cell proliferation in colon cancer cells. In turn, intestinal tumorigenesis may alter clock function as a result of increased β-catenin destabilization of PER2 protein and impairing circadian clock gene expression in intestinal mucosa of $Apc^{Min/+}$ mice[61].

In mice, mutation in the *Period* genes leads to altered temporal expression of genes involved in cell-cycle regulation and tumor suppression, such as *c-Myc*, *Cyclin D1*, *Cyclin A*, *Mdm-2* and *GADD45A*, deregulation of DNA-damage response, accelerated intestinal polyp formation in $Apc^{Min/+}$ mice, and increased neoplastic growth[62]. Moreover, the *BMAL1* gene is a fundamental hinge in the clock gene machinery and plays a key role in the regulation of tumor cell apoptosis, cell-cycle progression, and DNA damage response. In *in vitro* and *in vivo* experiments the knockdown of *BMAL1* by RNA interference in murine colon cancer cells (C26) reduced the expression of *Per1, Per2, Per3, Wee1* and *Tp53*; decreased apop-

tosis induced by etoposide; reduced the distribution in the G2/M phases of cells treated by docetaxel; and decreased DNA damage induced by cisplatin, accelerating cell proliferation *in vitro* and promoting tumor growth in mice[63]. A role in the early stages of colorectal carcinogenesis is played also by CK1ε, as demonstrated by *in vitro* and *in vivo* studies, and knock down of *CSNK1E* or use of a kinase inhibitor specific to CK1ε induced tumor-cell-selective cytotoxicity, because tumor cells depend more on the kinase activity of CK1ε than normal cells do[64,65].

The function of the circadian clock during neoplastic transformation was evaluated in a mouse model of chemically induced primary CRC, and the daily profiles of the core clock genes *Per1*, *Per2*, *Rev-Erba* and *Bmal1*, the clock-controlled gene *Dbp* and the clock-controlled cell cycle genes *Wee1*, *c-Myc* and *p21* were assessed in the tumor, matched nontumorous tissue, and the liver[66]. The circadian rhythmicity of *Per1*, *Per2*, *Rev-Erba* and *Dbp* was significantly decreased in CRC compared with nontumorous tissue, and the expression of *Bmal1* was not rhythmic. Besides, the circadian expression of *Per1*, *Per2*, *Rev-Erba* and *Dbp* was present in the nontumorous matched colonic tissue, but the expression of *Bmal1* did not show a circadian rhythm[66]. The expression patterns of *Wee1*, *c-Myc* and *p21* did not show circadian rhythmicity in tumors or the colon of healthy animals. A phase shift was evidenced for the rhythmic expression of the clock genes in the liver of tumor-bearing mice[66].

Clock genes in human colorectal cancer

There has been much interest in the alteration of expression of clock genes and clock-controlled genes in humans affected by CRC[67,68]. *CLOCK* is reported to be mutated in cancer, and may be involved in carcinogenesis through changed response to DNA damage. Microarray gene expression data combined with public gene sequence information have identified *CLOCK* as a potential target of somatic mutations in microsatellite unstable CRCs, and *CLOCK* mutations occurred in 53% of MSI CRCs[69]. Restoring *CLOCK* expression in a human colon adenocarcinoma cell line derived from a primary colon cancer lacking wild-type *CLOCK*, and testing the effects of UV-induced apoptosis and radiation by DNA content analysis using flow cytometry, demonstrated protection against UV-induced apoptosis and decreased G_2/M arrest in response to ionizing radiation. Novel CLOCK-binding elements were identified near DNA damage genes *p21*, *NBR1*, *BRCA1* and *RAD50* by means of chromatin immunoprecipitation with parallel DNA sequencing[69]. Furthermore, changes in the expression of PER2 were demonstrated by immunohistochemical staining and real-time polymerase chain reaction in tumor tissue of CRC patients, with heterogeneous, and negative or weak staining patterns in cancerous cells, with higher PER2 expression in well-differentiated cancer cells when compared to poorly differentiated ones, and associations of decreased PER2 levels with patient age, histological grade, TNM stage and expression of nuclear proliferation-related antigen Ki67[70]. Besides, downregulation of *PER3* was seen in tumor tissues of CRC patients, associated with various clinicopathological factors, comprising tumor location, differentiation, and stage, and to poorer survival, suggesting an important role in CRC progression[71]. Among the clock-controlled genes that drive intestinal physiology, an important role is played by *PPARG*, which codes for the transcription factor peroxisome proliferator-activated receptor (PPAR)γ, which is involved in several physiological processes, and an important interplay has been demonstrated between PPARγ and β-catenin[72]. β-Catenin is crucial in the WNT signaling pathway, and plays an essential role in the regulation of gene expression interacting with several molecular partners, such as APC and the TCF/LEF1 family transcription factors, and in cell adhesion, bridging between cadherins and the actin cytoskeleton at the cell-cell adherens junctions. Cytoplasmic but not membrane-bound β-catenin forms a complex with APC, the associated protein axin/conductin, CK2 and GSK-3β, which phosphorylate β-catenin and other elements of the molecular proteolytic complex. Activation of WNT signaling involves the inhibition of GSK-3β through mechanisms that may involve axin binding to the proteins Dishevelled or low-density lipoprotein receptor-related protein (LRP)-5, determining nuclear β-catenin accumulation, binding to TCF/LEF1 transcription factors, and expression of target genes including c-*MYC* and *CCND1*[73]. The protein kinase CK1ε is involved in cell proliferation by stabilizing β-catenin, and CK1ε overexpression mimics the effect of WNT signalling, resulting in cytoplasmic accumulation of β-catenin and its subsequent nuclear localization, to control transcription and support tumorigenesis[74-76]. The role of CK1ε in cell proliferation and in circadian clock function in the context of colorectal carcinogenesis might be not crucial. In the absence of WNT signaling, β-catenin is destabilized by GSK-3β, which plays a role in mammalian circadian clock function, counteracts the ability of CK1ε to promote β-catenin stability, and is involved in circadian control of cell-cycle progression[77].

PPARγ is a ligand-activated transcription factor belonging to the large superfamily of nuclear receptors, regulates cellular proliferation/differentiation, and plays a role in colorectal carcinogenesis[78]. In the normal mucosa of the colon and rectum there is high PPARγ expression, and in mouse small intestine and colon, decreased intestinal PPARγ levels are associated with enhanced tumorigenicity[79]. In CRC patients, PPARγ expression levels are not uniformly changed, suggesting that *PPARG* deregulation plays different and context-dependent roles in CRC onset and progression, and that this nuclear receptor inhibits tumor growth only in the presence of functional APC[80].

The functioning of the clock gene machinery is severely altered in patients affected by CRC, with downregulation of *BMAL1*, *PER1*, *PER2*, *PER3* and *CRY2*,

upregulation of *BMAL2* and *TIMELESS* (in particular in MSI-H and MSI-L tumors), and significant differences in survival related to differential expression of clock genes[44,81]. The altered expression of clock genes influences also phenotypic characteristics of colon cancer cells and disease outcome, and a correlation between high expression of the *BMAL1* gene and low expression of the *PER1* gene and liver metastasis, and between high expression of the *PER2* gene and significantly better outcomes of CRC have been observed[82]. The upregulation of *BMAL2* is accompanied by high expression of *SERPINE1* in CRC patients[81]. Invasion by cancer cells requires proteases to degrade the cellular matrix. Cathepsins, which are cysteine proteases, play a crucial role in this process through the destruction of the cell-surrounding extracellular matrix[83]. Plasmin is formed from its zymogen, plasminogen; a reaction catalyzed by the serine protease urokinase-type plasminogen activator (uPA) and partially regulated by plasminogen activator inhibitors, such as plasminogen activator inhibitor (PAI)-1[84]. A high concentration of PAI1 in tumor biopsy specimens, the major physiological regulator of the pericellular plasmin-generating cascade, is associated with poor prognosis, and high preoperative plasma concentrations of PAI-1 are associated with shorter survival in CRC patients[85]. Both CLOCK:BMAL1 and CLOCK:BMAL2 heterodimers powerfully activate the promoter of the gene encoding PAI-1, officially called *SERPINE1* and located on the seventh chromosome (7q21.3-q22), underlying the circadian variation in circulating PAI-1[86,87]. CLOCK:BMAL2, binding two E-box enhancers in the promoter, is more potent than CLOCK:BMAL1 in its ability to activate *SERPINE1*[88-90].

Colorectal tumorigenesis is influenced also by SIRT1 that may behave as a tumor suppressor or as an oncogene in different *in vitro* and *in vivo* conditions. The involvement of SIRT1 in onset and progression of CRC is corroborated by the observation that transgenic mice overexpressing SIRT1 in the intestine show reduced development of tumors caused by $Apc^{Min/+}$ mutation, and that $SIRT1^{-/-}$ mice show increased tumor incidence when crossed to a $p53^{-/-}$ background. In CRC patients, the expression levels are unevenly modified, reflecting the different settings that characterize the experimental conditions, and different colon cancer cell lines show dissimilar levels of *SIRT1* expression and variable changes in clock gene expression after ectopic upregulation[91]. In normal colon, significant levels of nuclear SIRT1 are expressed in proliferating colon epithelial cells at the base of the crypt, and expression gradually decreases in cells migrating toward the lumen. In contrast, in samples of benign adenomas (polyps), SIRT1 is detected in all cells with an adenomatous morphology of benign adenomas (polyps), but not in the adjacent normal mucosa. Colonic adenocarcinoma shows a heterogeneous SIRT1 expression profile, suggesting that in normal colon epithelium, SIRT1 levels correlate with active cell proliferation. SIRT1 is expressed at high levels in normal colon and benign lesions, and high SIRT1 expression is an intrinsic response to cell proliferation in untransformed mucosa and premalignant adenoma. In CRC tissue, SIRT1 expression is downregulated in a subset of tumors to facilitate tumor growth, and in particular in high grade tumors, where the growth-inhibitory activity of SIRT1 becomes a rate-limiting factor for tumor progression, whereas some low- grade tumors may overexpress SIRT1 to benefit from its antiapoptotic effects[92].

Circadian rhythmicity and CRC treatment

The body systems involved in xenobiotic detoxification show circadian variations of function and activity driven by the clock gene machinery, determining time-dependent toxicity of xenobiotics and drugs[93-95]. The product of the multidrug resistance (*mdr1a*) gene, P-glycoprotein, works as a xenobiotic transporter contributing to the intestinal barrier, and intestinal expression of the *mdr1a* gene and its efflux pump function show oscillation with 24-h periodicity driven by the circadian clock through the opposing action of HLF and E4BP4, connecting the circadian timekeeping system to xenobiotic detoxification[96]. The time-of-day-dependent variations in cancer cell proliferation, as well as drug metabolism, toxicity and effectiveness represent the rationale for the timing of drug administration over a 24-h period[97-100], and they form the basis for chronomodulated chemotherapy of advanced-stage CRC, characterized by an opposite phase of delivery of oxaliplatin in the afternoon, with respect to 5-fluorouracil and leucovorin that are delivered late at night (chronoFLO4)[101]. Chronomodulated delivery of chemotherapy has shown better tolerability and antitumor activity compared with conventional chemotherapy, with less myelosuppression and more gastrointestinal toxicity for the chronoschedule, but with a favorable difference between treatments in median survival time only in male patients. Indeed, sex dependency in the effects of this schedule has been demonstrated with shorter survival and greater toxicity reported in female CRC patients in the European Organisation for Research and Treatment of Cancer (EORTC) Chronotherapy Group trial[102]. From these studies, sex emerges as the single predictor of survival, conditioning outcome of chronoFLO4 and determining genetic variation of metabolic responses that influence time-related variables and hinder administration of maximal effective dosing. A different genotypic profile between men and women could characterize CRC patients, and the higher burden of toxicity reported in women treated with 5-fluorouracil-based chemotherapy may be also due to the lower expression of dihydropyrimidine dehydrogenase in the tumors in female patients and/or to sex dependency of circadian pharmacology[68]. Xenobiotic detoxification and metabolic pathways differ between male and female mammals including humans. In double mutant $Cry1^{-/-}Cry2^{-/-}$ male mice, sex dimorphism in hepatic drug metabolism is altered. Upon inactivation of the Cryptochrome genes, the levels of sex-specific liver products, including several cytochrome P450 enzymes,

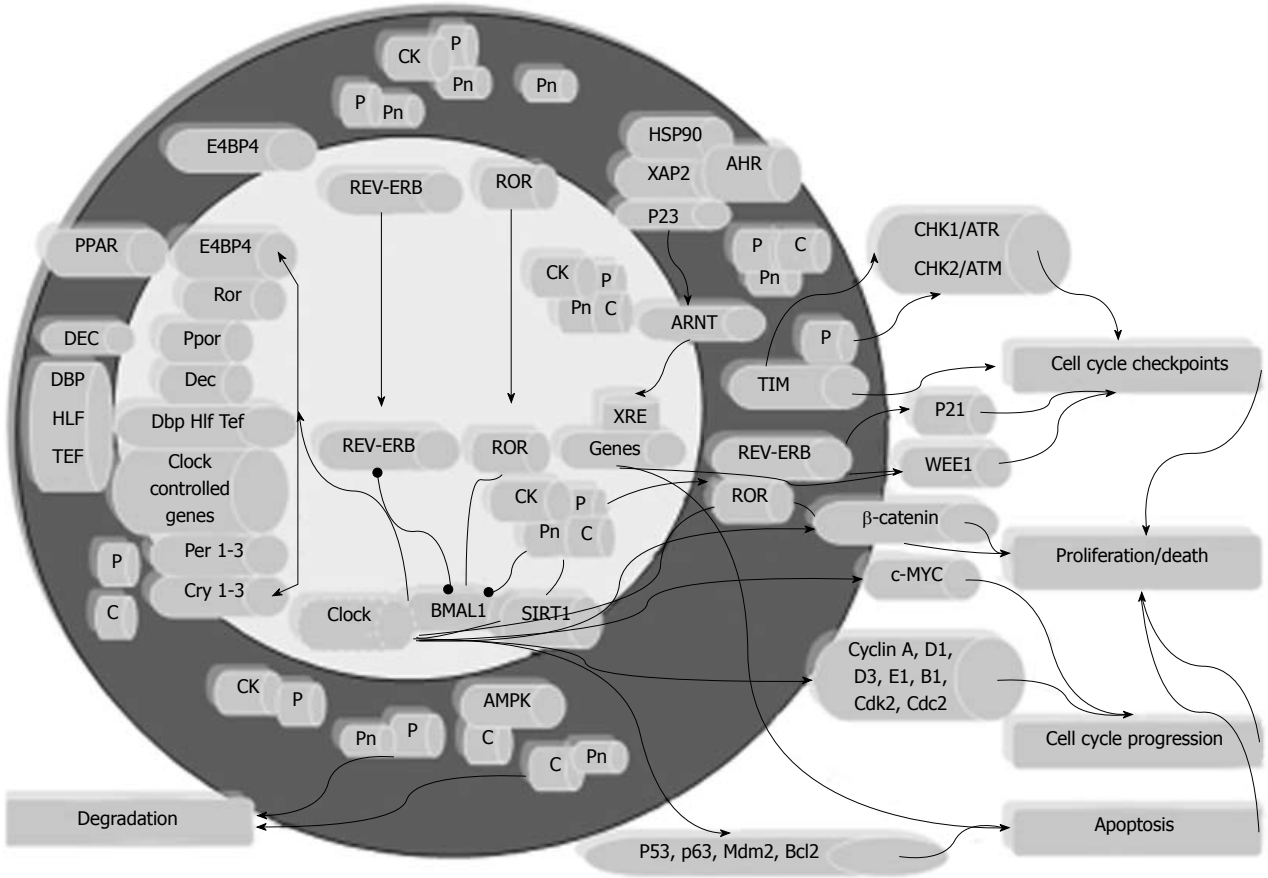

Figure 1 Biological clock and the cell processes involved in colorectal carcinogenesis. The scheme renders the transcriptional/translational feedback loop through which the molecular clockwork operates, and depicts its interplay with the aryl hydrocarbon receptor (AHR)/ARNT system in the control of the cell cycle and apoptosis, and ultimately in the regulation of the processes of cell proliferation and death, whose deregulation brings about colorectal cancer (CRC) onset and progression. CK: Casein kinase; AHR: Aryl hydrocarbon receptor; PPAR: Peroxisome proliferator-activated receptor; CHK: Checkpoint kinase; ATR: Ataxia telangiectasia and rad3-related; ATM: Ataxia telangiectasia mutated; ARNT: Aryl hydrocarbon receptor nuclear translocator; HLF: Hepatic leukaemia factor; TEF: Thyrotroph embryonic factor.

expressed by male mice are similar to those expressed by female mice[103]. In humans, the decreased expression level of Cryptochrome genes observed in female patients affected by CRC[44] might explain the different median survival and increased toxicity observed after chrono-modulated chemotherapy administration in the EORTC Chronotherapy Group trial[104]. High Cryptochrome gene expression and protein levels in tumor tissue are correlated with tumor progression and poorer overall and disease-free survival in CRC patients[104].

Many cytotoxic anticancer drugs damage DNA and may activate DNA checkpoints permitting attempted DNA repair. This process is essential for cell survival, but may reduce the cytotoxicity of anticancer drugs. TIMELESS is required for ATM-dependent Chk2-mediated signaling of doxorubicin-induced DNA double-strand breaks, and downregulation of *TIMELESS* by siRNA significantly attenuates doxorubicin-induced G_2/M cell cycle arrest and sensitizes cancer cells to doxorubicin-induced cytotoxicity. Therefore, upregulation of *TIMELESS* in CRC may predict altered drug sensitivity, and TIMELESS inhibition is a potential novel anticancer drug target to enhance the cytotoxic effectiveness of chemotherapeutic drugs known to activate DNA response pathways within cancer cells[105]. *TIMELESS* expression is also significantly associated with MSI status in CRC patients, and higher expression is found in patients with MSI-H and MSI-L[44]. Approximately 15% of CRC is characterized by defects in the DNA mismatch repair system, leading to MSI that generates a large number of substitution, as well as insertion and deletion mutations. These mutations typically target microsatellite sequences and lead to shifts in the reading frame, resulting in truncation or other alterations of the protein product. The presence of premature stop codons decreases the expression of mRNAs with such frameshift mutations and results in degradation of some of the mutant mRNA through the nonsense-mediated decay pathway. Response to adjuvant chemotherapy may be influenced by MSI status in CRC patients, because MSI-H tumors show better outcome with irinotecan-containing than 5-fluorouracil-containing regimens[106]. The increased expression of *TIMELESS* in MSI-H and MSI-L CRC may be related to the process of tumorigenesis and might cause reduced response to possible adiuvant chemotherapy. The circadian CLOCK-BMAL1 transactivation complex may be directly involved in the response to stress at the individual cell level, because *Clock* mutant and *Bmal1* knockout mice are highly sensitive to cyclophosphamide treatment in a time-of-day-independent manner, suggesting that CLOCK and BMAL1 directly regulate

the expression of cell-cycle- and apoptosis-related genes, and that time-related and allelic-dependent variations in response to chemotherapy correlate with the functional status of the CLOCK-BMAL1 heterodimer[93-95,107]. The frequencies of the *311T>C CLOCK* gene CC genotype and C allele were significantly higher among CRC patients compared to controls, increasing the risk of CRC by 2.78- and 1.78-fold, respectively[108]. Furthermore, higher levels of *CLOCK* gene and protein expression were observed in human CRC tissues, particularly in poorly differentiated, advanced Dukes' stage tumors and in cases with lymph node involvement, and a strong positive linear correlation was found with ARNT, hypoxia-inducible factor-1α and vascular endothelial growth factor expression in tumor tissue[109,110]. BMAL1 protein suppresses the proto-oncogene *c-MYC*, and stimulates the tumor suppressor *WEE1*, confirming the important role played by the altered expression of this core clock gene in the process of carcinogenesis[50]. In addition, a tight interplay has been demonstrated between aryl hydrocarbon receptor (AHR)/ARNT system and circadian pathways, and this interconnection might be critical to influence onset, promotion, and progression of colorectal malignant neoplasms[46]. An important role is played in carcinogenesis by the transcription factors of the AHR/ARNT system, which bind to a xenobiotic response element and control the expression of genes encoding enzymes involved in the metabolism and detoxification of exogenous and endogenous compounds, but they are antagonized by the AHR repressor[111] (Figure 1). Upon ligand-mediated activation, the AHR/ARNT system modulates metabolic pathways and cell processes, such as survival, cell cycle, proliferation, apoptosis, differentiation, epithelial to mesenchymal transition, angiogenesis, inflammation, cell contact inhibition, cell-matrix interaction, and extracellular matrix remodeling, which are crucial in the initiation and evolution of neoplastic disease[112-114].

CONCLUSION

The biological clock plays an important role in bowel physiology, and alteration of the molecular clockwork is involved in colorectal carcinogenesis. The circadian genes and proteins are variably altered in colorectal malignant neoplasm and influence phenotypic characteristics of colon cancer cells, disease progression, patient survival, and response to chemotherapy. A better understanding of the mechanisms controlled by the clock gene machinery in normal conditions and in time- and biological-clock-related derangements in colorectal carcinogenesis will allow further advances in the comprehension of the pathophysiological mechanism operating in intestinal cancer, and will provide more effective therapeutic strategies for patients suffering from colorectal malignant tumors.

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